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Heather Mernitz ^a; Fuzhi Lian ^a; Donald E. Smith ^a; Simin Nikbin Meydani ^a; Xiang-Dong Wang ^a

^a Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA

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Fish Oil Supplementation Inhibits NNK-Induced Lung Carcinogenesis in the A/J Mouse

Heather Mernitz, Fuzhi Lian, Donald E. Smith, Simin Nikbin Meydani, and Xiang-Dong Wang

Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA

High intake of fish oil with a low omega-6 (n-6)/omega-3 (n-3) polyunsaturated fatty acid (PUFA) ratio has been suggested to protect against many chronic diseases. However, the effect of different ratios of dietary n-6 and n-3 PUFA on lung tumorigenesis has not been investigated. In this study, we examined the effect of a 4 mo dietary supplementation with corn oil (with a high n-6/n-3 ratio) and fish oil (with a low n-6/n-3 ratio) as compared with soybean oil (isocaloric control with the same n-6/n-3 ratio as the base diet) on tumor incidence and tumor prevalence in the A/J mouse model of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung carcinogenesis. We found that dietary supplementation had no effect on overall lung tumor incidence, but fish oil supplementation was able to decrease lung tumor prevalence by 78% and 80% compared to groups receiving soybean oil and corn oil supplementation, respectively. The inhibitory effect of fish oil on lung tumor prevalence was associated with increased expressions of cell cycle inhibitor p21Cip1 and lipoxygenase isoform 15-LOX in the lungs. These data suggest that fish oil with a low ratio of n-6/n-3 PUFA could be beneficial in the prevention of lung carcinogenesis.

INTRODUCTION

Lung cancer is the most common cancer and the leading cause of cancer death both globally and in the United States. Despite great efforts to improve smoking cessation and the treatment of patients with lung cancer, the survival rate for people diagnosed with this disease has not significantly improved over the past 30 yr, which underscores dietary intervention as one of the main strategies for preventing lung cancer. Although its preventive role against cancer has yet to be conclusively

established (1), high intake of fish oil/omega-3 polyunsaturated fatty acids (n-3 PUFA) has been suggested to protect against many chronic diseases including cancers (2–5). A significant inverse correlation between fish consumption and lung cancer mortality was found in men in countries with high levels of cigarette smoking or animal fat consumption, such as Iceland and Japan (6). In case-control studies, a retrospective case-control study among Hong Kong Chinese women who never smoked tobacco found that those in the lowest tertile of consumption of fresh fish had a statistically significant adjusted relative risk of 2.8 for developing lung cancer (7); and a comparison of 71 consecutive, new male cases of lung cancer and 71 male hospital control patients showed a significantly lower intake of fish in lung cancer patients (8). In prospective studies, a study in Norway involving 51,452 subjects and 154 lung cancer cases noted a significant protective effect of cod liver oil (rich in n-3 PUFA) against lung cancer (9); and a Japanese prospective study showed that frequent consumption of fresh fish or shellfish was associated with lower lung cancer risk (10). However, other studies have shown fish intake was not associated with either lung cancer risk (11) or lung cancer death (12). One explanation for these inconsistent results may be confounding by varying intakes of other fatty acids, such as omega-6 polyunsaturated fatty acids (n-6 PUFA) from vegetable oil. Studies have indicated that in contrast to the cancer preventive effect of n-3 PUFA, n-6 PUFA such as linoleic acid (LA) is associated with higher risk of cancer (13). This leads to the hypothesis that the ratio of dietary n-6 to n-3 PUFA may be the most important factor in determining cancer risk relative to fatty acid intake. This notion is supported by the observation that the growth of implanted melanoma cells and lung cancer cells was dramatically inhibited in mice whose n-6/n-3 fatty acids were genetically decreased (14,15), whereas the lung tumor volume was increased in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-treated rats fed with dietary corn oil from 5% to 23.5% (16). However, the effect of different ratios of dietary n-6 and n-3 PUFA on carcinogen-induced lung tumorigenesis has not been investigated.

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Address correspondence to Xiang-Dong Wang, Nutrition and Cancer Biology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111. Phone: 617-556-3130. Fax: 617-556-3344. E-mail: xiang-dong.wang@tufts.edu

One explanation for the anticarcinogenic activity of n-3 PUFA is their ability to modulate arachidonic acid (AA) metabolism (17). AA is a predominant fatty acid in cell membranes. It is a metabolite of LA, a major n-6 PUFA in vegetable oils such as corn oil and soybean oil, generated by desaturation and elongation. α -linolenic acid (ALA), a major n-3 PUFA in vegetable oil, shares the same metabolic pathway and generates eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are also available from fish oil. Therefore, dietary LA and ALA may compete with each other for metabolizing enzymes. Furthermore, AA, EPA, and DHA all serve as substrates for cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to generate eicosanoids that can affect diverse biological processes (17,18). COX enzyme products give rise to prostaglandins and thromboxanes, whereas LOX enzymes produce leukotrienes, hydroxyfatty acids, and lipoxins. In general, n-6 PUFA-derived eicosanoids seem to have proinflammatory effects and have been linked to carcinogenesis, whereas n-3 PUFA-derived eicosanoids have been associated with anti-inflammatory effects (19–21). Therefore, n-3 PUFA including ALA, EPA, and DHA can compete with n-6 PUFA such as LA and AA for enzyme availability, resulting in an altered response to cancer cells and modulation of inflammation, cellular proliferation, apoptosis, metastasis, and angiogenesis (17,19). In addition, n-3 PUFA may also directly affect the expression of fatty acid metabolizing enzymes. For example, EPA and DHA were shown to inhibit cytokine-induced COX-2 gene transcription *in vitro* and *in vivo* (22,23). There have been no reports of the effect of n-3 PUFA supplementation on the expression of LOX and COX2 enzymes, so this remains an area of research interest.

Previously, it has been shown that in Lewis lung carcinoma-bearing C57BL/6 mice, a diet containing 5% fish oil resulted in significantly slower growth of primary tumors, lower mortality rate, and lower metastatic spread compared with tumor-bearing mice on a diet containing 5% soybean oil (24). However, no studies have been conducted to examine whether high intake of omega-3 PUFA or a diet containing low ratio of n-6 to n-3 PUFA can affect lung tumorigenesis. Using the A/J mouse model of NNK-induced lung carcinogenesis (25), a widely used animal model for lung cancer, we have shown the potential chemopreventive effect of dietary supplementation with 9-cis-retinoic acid (26), $1\alpha,25$ -dihydroxyvitamin D₃ (27), and lycopene (28) against lung carcinogenesis. The goal of this research was to determine whether oils with differing content of n-6 and n-3 PUFA could exert measurable effects on tumor incidence and tumor prevalence in the A/J mouse model of lung cancer. Furthermore, we determined whether dietary supplementation with different ratios of n-3/n-6 PUFA could alter gene expression of COX-2, LOX enzymes (5-LOX, 8-LOX, 12-LOX, or 15-LOX), and several cell cycle regulators (p21, p27, Cyclin D, or Cyclin E) in the mouse lung.

MATERIALS AND METHODS

Polyunsaturated Oils

Menhaden fish oil (donated by Omega Protein, Inc., Reedville, VA) contains approximately 30.5% total n-3 PUFA (including 12.9% of EPA, 8.17% of DHA, and 2.03% of ALA) and 5.1% total n-6 PUFA (including 1.73% of LA and 0.22% of AA). Corn oil (Crisco[®] pure corn oil) and soybean oil (Mazola[®] 100% vegetable oil) were purchased commercially. Based on USDA national nutrition database values, corn oil contains 53.5% of n-6 PUFA (predominantly LA) and 1.2% of n-3 PUFA as ALA, whereas soybean oil contains 51.0% of n-6 PUFA mainly as LA and 6.8% of n-6 PUFA as ALA. The n-6/n-3 PUFA ratios for fish oil, soybean oil, and corn oil used are estimated as 0.2, 7.5, and 46.1, respectively.

Animal Model and Experimental Design

Male A/J mice (6 wk old) obtained from Jackson Labs (Bar Harbor, ME) were randomly assigned to 1 of 5 experimental groups ($n = 14$ animals/group). Each group was fed AIN-93M semipurified diet (Dyets; Bethlehem, PA) for 2 wk and then switched over to experimental diets as follows:

- Group I (Sham + Control): Control diet (AIN-93M) plus i.p. injection of 0.20 ml normal saline/kg body weight
- Group II (NNK + Control): Control diet plus ip injection of 100 mg NNK/kg body weight
- Group III (NNK + Fish oil): Control diet supplemented with menhaden fish oil (10%) plus ip injection of 100 mg NNK/kg body weight
- Group IV (NNK + Corn oil): Control diet supplemented with corn oil (10%) plus ip injection of 100 mg NNK/kg body weight
- Group V (NNK + Soybean oil): Control diet supplemented with soybean oil (10%) plus ip injection of 100 mg NNK/kg body weight

Previous work has shown that a single intraperitoneal injection of 2 mg or 100 mg/kg body weight of NNK was sufficient to induce tumors in 100% of A/J mice at a level of 10 to 12 adenomas per mouse at 16 wk postinjection (25,26,29). In this study, treatment of A/J mice with oil diets began 3 wk prior to intraperitoneal injection of 100 mg NNK/kg body weight (Toronto Research Chemicals, Inc., Ontario, Canada).

The AIN-93M base diet contains 4% soybean oil (wt/wt), and oil-supplemented groups received 10% (wt/wt) fish oil, corn oil, or soybean oil in addition to the oil in the base diet. Thus, oil-supplemented groups were all maintained on 14% fat diets, but each diet had a different n-6/n-3 fatty acid ratio. The corn oil-supplemented diet had a high n-6/n-3 fatty acid ratio; the fish

oil-supplemented diet had a low n-6/n-3 fatty acid ratio; and the soybean oil-supplemented diet served as an isocaloric control, with the same n-6/n-3 fatty acid ratio as the base diet but the higher caloric density of the experimental diets. Oils were mixed directly into powdered AIN-93M diet. Oil supplemented diets were prepared in 2 kg batches once a month and stored in 500 g airtight bags under nitrogen blanket at -20°C to delay oxidative decomposition of the oils. Once opened, diets were used for 1 wk, giving animals fresh portions daily and storing the remainder at 4°C under nitrogen blanket. Because the fish oil was supplemented with vitamin E by the manufacturer to prevent oxidative degradation, vitamin E (27 mg/kg diet, all-*rac*- α -tocopherol acetate; Roche Vitamins, Inc., Parsippany, NJ) was added to the corn and soybean oils to maintain similar levels among the 3 oil-supplemented diets to control for potential antioxidant effects on carcinogenesis.

Mice were maintained on study diets for 17 wk postinjection, fed ad libitum, observed daily, and weighed weekly. Mice were fasted for at least 12 h prior to terminal exsanguination under deep anesthesia (5% isoflurane; Baxter Healthcare Corp., Deerfield, IL), and blood was collected and stored as plasma. The right lungs were perfused with buffered Formalde-Fresh solution (Fisher Chemicals, Fairlawn, NJ) and maintained in Formalde-Fresh until sectioning and slide preparation. The left lungs were snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Quantitation of Lung Lesions

Lung lesions were quantified by determining the incidence and prevalence of pulmonary pleural surface tumors, counting both the right and left lungs of each mouse. Tumor incidence was defined as the number of lung sets (right and left lungs) in each

group containing 1 or more tumors divided by the total number of lung sets examined. Tumor prevalence (mean number of tumors per mouse) was defined as the total number of pulmonary tumors in each group divided by the total number of lung sets examined. Lung tumors were visually counted by two researchers blinded to the treatment group, and lung sections were microscopically examined to confirm presence of bronchioalveolar adenoma.

Real-Time PCR Analysis

Total RNA was isolated from lung tissue using RNeasy Mini Kits (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. cDNA was prepared from the RNA samples using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and an automated thermal cycler (MJ Research PTC-200; Bio-Rad Laboratories, Hercules, CA) and quantified using a fluorescence-based, real-time detection method (ABI PRISM 7000 Sequence Detection System; Perkin-Elmer Applied Biosystems, Foster City, CA) using SYBR Green reagents (Applied Biosystems, Foster City, CA). The PCR reaction was carried out in 20 μl of reaction mixture containing 10 μl of Power SYBR Green PCR Mastermix, 1 μl of 10 μM primer mix (including forward and reverse primers for target genes or an internal reference gene, β -actin), and 9 μl (25 ng) of cDNA diluted in RNase-free water. Cycling conditions were 50°C for 2 min and 95°C for 2 min followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. Melting curves were run following the cycling program to confirm the specificity of the reaction for the amplicon. Sequences for primers are as follows (Sigma-Genosys, The Woodlands, TX):

The primers sequences for p27, cyclin D, and E are as described (28). Relative standard curves and relative efficiency

Gene	Accession Number	Primer Sequence	
p21Cip1	NM_001111099	forward primer	ATTCAGAGCCACAGGCACCAT
		reverse primer	TCAAAGTTCCACCGTTCTCGG
COX-2	NM_011198	forward primer	GCTGCCCGACACCTTCAA
		reverse primer	TTCTTCCCCCAGCAACCC
5-LOX	NM_009662	forward primer	CTGAGGTGTTTGGTATCGCCAT
		reverse primer	GGCATTGGCCTTGTCAAAAAG
8-LOX	NM_009661	forward primer	AGTTCAGGCCAGTTCGACTCTT
		reverse primer	CAGCCAGAGAGCAATGATGTG
12-LOX	NM_007440	forward primer	CGGCCATGTTTCAGTTGCTTACT
		reverse primer	ATCCCTTCACATACCTGGCA
15-LOX	NM_009660	forward primer	AGCTCATTGTGTCCCCCTGAT
		reverse primer	ACATTCCCACCACGTACCGAT
β -actin	NM_007393	forward primer	TAGACTTCGAGCAGGAGATGGC
		reverse primer	CCACAGGATTCCATACCCAAGA

plots were constructed and verified over the working range of mRNA levels to confirm that amplification efficiencies of the genes were approximately equal to the endogenous control gene, β -actin. Quantification of gene expression was calculated relative to average values for the Sham control group using the comparative Ct method. For each sample and each gene, PCR reactions were carried out in duplicate.

Statistical Analyses

A student *t*-test was used to compare means between sham + control and NNK + control groups (with same diet but different NNK treatment) and between NNK + control and NNK + soybean oil groups (with same NNK treatment and same ratio of n-6/n-3 but receiving different amount of oil in the diet). A one-way ANOVA analysis with Fisher's least significant difference (LSD) method was used to compare means across oil-supplemented groups (receiving same amount of oils but with different n-6/n-3 ratio). A difference was considered significant at *P* < 0.05.

RESULTS

Effects of Fish Oil and Corn Oil on Body Weight and Lung Tumors

There were no significant differences in mean body weight between groups at baseline or at the end of dietary supplementation period (Table 1). This suggests that animals were able to adjust dietary intake relative to the caloric density of the diet. There were no tumors present in any of the lungs examined from the sham plus control group at 16 wk. Although spontaneous lung tumors can form in A/J mice, the incidence of spontaneous tumors in mice 25 wk old is very low, so a lack of tumors in this group was not surprising (30). Compared to this group, the groups receiving NNK alone or NNK plus different oils had significantly higher tumor incidence and tumor prevalence (*P* < 0.001 for both, Table 2). No difference in tumor incidence and prevalence was found between mice receiving NNK plus soybean oil (containing 14% soybean oil)

TABLE 2
The effects of oil supplementation on lung tumor measures^a

Treatment Group	Tumor Incidence	Tumors/Mouse
Sham + control	0/13	0
NNK + control	14/14 (100%)	15.50 ± 2.47
NNK + soybean oil	14/14 (100%)	14.14 ± 4.91
NNK + fish oil	14/14 (100%)	3.14 ± 1.56 ^b
NNK + corn oil	14/14 (100%)	15.79 ± 2.55

^aAbbreviation is as follows: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Means ± SD are shown. *n* = 13 to 14 animals per group.

^bSignificant difference as compared with other oil-supplemented groups at *P* < 0.001.

and mice receiving NNK treatment and fed a base diet (containing 4% soybean oil) diet (Table 2). Compared to the soybean oil group, neither fish oil nor corn oil supplementation affected tumor incidence (100% in all 3 groups); however, animals in the fish oil supplemented group had significantly lower tumor prevalence (78% lower, *P* < 0.001) (Table 2). The size of the lung tumors among the groups were fairly consistent (between 0.8–1.2 mm), and only a few slightly larger tumors (≈ 2 mm) were observed in the current study and in our previous studies (26–28).

Effects of Fish Oil and Corn Oil on Lung Expression of LOX and COX2 Enzymes

The effect of dietary n-6 and n-3 PUFA on the expression of COX and LOX enzymes were compared among groups of mice supplemented with corn, soybean, and fish oil. There were no significant differences in COX-2, 5-LOX, or 8-LOX mRNA levels among the 3 groups (Table 3). Fish oil supplementation significantly increased mRNA levels of 12-LOX and 15-LOX by 58% and fivefold, respectively, as compared with mice fed with soybean oil (Table 3). There was no significant difference in 12-LOX and 15-LOX mRNA between corn oil and soybean oil groups (Table 3). Neither NNK treatment nor increased dietary fat intake significantly affects expression of COX-2 and LOX enzymes in mouse lung tissues (Table 3).

Effect of Fish Oil and Corn Oil on Lung Expression of Cell Cycle Regulators

Higher mRNA levels of p21, a cell cycle inhibitor gene, were observed in the lung tissues of mice fed fish oil as compared with mice fed soybean oil and corn oil (Table 3). Neither NNK treatment nor increased dietary fat intake significantly affects p21Cip1 expression in mouse lung tissues (Table 3). No significant changes in the expression levels of other genes regulating cell growth or survival, such as p27Kip1, cyclin D, and cyclin E, were detected in the lung tissues of mice treated with NNK or supplemented with different oil (data not shown).

TABLE 1
The effects of feeding different oils on mouse body weight^a

Treatment Group	Body Weight at Baseline (g)	Body Weight at Sacrifice (g)
Sham + control	19.86 ± 2.27	23.04 ± 2.71
NNK+ control	19.84 ± 1.39	23.36 ± 1.98
NNK + soybean oil	19.89 ± 1.13	25.20 ± 2.88
NNK + fish oil	19.89 ± 1.72	22.99 ± 2.62
NNK + corn oil	19.84 ± 1.10	24.18 ± 3.48

^aAbbreviation is as follows: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Means ± SD are shown. *n* = 13 to 14 animals per group (*n* = 14 animals per group for baseline body weight).

TABLE 3
The effects of oil supplementation on gene expression measures^a

Treatment Group	5-LOX	8-LOX	12-LOX	15-LOX	COX-2	p21
Sham + control	1.13 ± 0.74	1.07 ± 0.39	1.04 ± 0.35	1.38 ± 1.91	1.08 ± 0.40	1.03 ± 0.31
NNK + control	0.86 ± 0.23	2.47 ± 1.23	1.11 ± 0.39	1.98 ± 2.12	0.80 ± 0.37	1.14 ± 0.42
NNK + soybean oil	1.21 ± 0.42	2.78 ± 1.30	0.98 ± 0.26	0.60 ± 0.52	0.86 ± 0.26	1.27 ± 0.52
NNK + fish oil	1.10 ± 0.38	2.89 ± 1.41	1.45 ± 0.35 ^b	3.55 ± 4.89 ^b	0.70 ± 0.34	2.31 ± 0.74 ^b
NNK + corn oil	1.08 ± 0.47	2.41 ± 1.06	1.07 ± 0.38	0.38 ± 0.28	0.75 ± 0.27	1.57 ± 0.48

^aAbbreviations are as follows: LOX, lipooxygenase; COX, cyclooxygenase; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Means ± SD are shown. *n* = 13 to 14 animals per group.

^bSignificant difference as compared with NNK + soybean oil, isocaloric control, *P* < 0.001.

DISCUSSION

In the present study, we determined the effect of dietary supplementation with 10% fish oil (with higher levels of EPA and DHA and low n-6/n-3 ratio) and 10% corn oil (with higher levels of LA and high n-6/n-3 ratio) as compared with supplementation with 10% soybean oil, a similar source of caloric density that maintains the n-3/n-6 PUFA ratio of the base diet that contains 4% soybean oil, on tumor incidence and tumor prevalence in the A/J mouse model of lung cancer. Our results showed that supplementation with 10% fish oil (wt/wt) resulted in significantly lower lung tumor prevalence in A/J mice 4 mo after carcinogen injection. Whereas previous studies have shown that supplementation with fish oil (31) or EPA/DHA (32) significantly inhibited the growth of xenografted tumors in mouse models, this is the first study to show that fish oil decreases lung tumor development in vivo in a carcinogen-induced lung cancer model, suggesting a preventive effect of n-3 PUFA against lung tumorigenesis. This study also reinforces the ratio of dietary n-6 to n-3 PUFA as the important factor in dietary chemoprevention.

It has been suggested that the beneficial effect of n-3 PUFA can be attributed to long chain fatty acids, predominant EPA and DHA (17). When compared with soybean oil and corn oil, fish oil contains much higher levels of EPA and DHA, whereas the major n-3 PUFA in corn oil and soybean oil is ALA, which is incorporated to phospholipids and then converted to EPA and DHA after absorption. However, the estimated efficiency for the conversion of ALA into EPA in humans only ranges between 0.2% and 8% (33). Therefore, supplementation with fish oil will result in much higher levels of EPA and DHA in mouse tissues as compared with corn oil and soybean oil supplementation, which may account for the significant lower tumor numbers in lung tissue of fish oil-supplemented mice.

In the current study, although we were able to show that supplementation with fish oil provides chemopreventive effects against lung carcinogenesis, we did not observe any promoting effects associated with supplementation with corn oil. This is in contrast to earlier studies that have shown that diets enriched in corn oil or LA increased tumor growth and development in rodent models of breast (34,35), colon (36,37), and prostate cancer (38,39). With respect to lung cancer, a diet enriched in corn

oil (20% vs. 5%) was shown to increase lung tumor volume and reduce tumor latency in F344 rats treated with NNK (16). One explanation for our conflicting results lies in the model we used in the study. With NNK-injected control animals developing an average of 15.5 lung tumors per mouse, it is possible that promoting factors may not be able to induce additional tumors beyond this level. Therefore, we can not exclude the possibility that the ratio of n-6/n-3 PUFA in corn oil could influence the process of lung carcinogenesis. Further study using either a lower dosage of carcinogen or examining earlier time points in the process of tumor initiation and promotion may address the issue.

Both omega-3 and omega-6 fatty acids are substrates for COX and LOX enzymes to generate biologically active eicosanoids. Several distinctive isoforms of LOX enzymes have been identified in mice and in humans, which stereo-specifically insert oxygen into polyunsaturated fatty acids and generate 5-, 8-, 12-, or 15-hydroperoxyeicosatetraenoic acids (HPETE) and leukotrienes (40). In the present study, we showed that fish oil supplementation significantly increased mRNA levels of 15-LOX and, to a much lesser extent, 12-LOX; whereas mRNA levels of COX-2, 5-LOX, and 8-LOX were not affected by oil supplementation. The increased expression of 15-LOX was associated with decreased NNK-induced lung tumor numbers. Recently, 15-LOX-1 and 15-LOX-2 (corresponding to 8-LOX in mouse) have been suggested to suppress tumorigenesis (40), whereas 5-LOX and 12-LOX are generally considered procarcinogenic. For example, 15-LOX is preferentially expressed in normal tissues and benign lesions and less in malignant tissues as well as in cancer cell lines (41–43). The overexpression of 15-LOX has been shown to inhibit cell growth and induce apoptosis in colon and pancreatic cancer cells (43,44). Furthermore, it has been shown that 15-LOX mediates nonsteroidal, anti-inflammatory, drug-induced apoptosis in colon cancer cells (45) and gastric cancer cells (46). Thus, the higher expression of 15-LOX in fish oil fed mice might have contributed to the lower prevalence of lung tumors in this study. The expression of 12-LOX has been shown to increase in various epithelial cancers including prostate, breast, and pancreatic cancer (47–49), and the inhibition of 12-LOX was shown to induce cancer cell apoptosis (50) and inhibit tumor

promotion (51). Since the fish oil-induced increase in 12-LOX was much less than that in 15-LOX, the significance of observed effect in 12-LOX in this study is questionable.

Although having significantly lower tumor prevalence in the group receiving fish oil supplementation, we did not detect any significant changes in COX-2 message or protein levels (52–54). One explanation for this could be differences in the method of NNK administration. Continuous administration of NNK in the drinking water may increase COX-2 expression (52), whereas a single dose of NNK may result in acute changes in gene expression that do not persist over the duration of the study (26). Further, the lack of significant change in the expression of COX-2 gene may be explained by altered COX-2 activity without necessarily altering protein levels. An effect on decreased enzyme activity and reduced prostaglandin E₂ production could not be confirmed in our in vivo study due to analytical limitations on frozen tissue samples. In the present study, the significantly lower tumor prevalence in the group receiving fish oil supplementation was associated with increased p21 mRNA expression in lung tissues. p21, the product of the CIP1/WAF1/SDI1 gene, was shown to inhibit G1 cyclin/cyclin-dependent kinase complexes and acts to constrain the cell cycle and facilitate tumor growth arrest. It has been shown that expression of p21 is a significant factor in predicting a favorable prognosis in patients with non-small-cell lung cancer (55). Our result is in agreement with a recent report that the fish oil supplementation enhanced p21 expression, which was associated with a decrease in aberrant crypt formation in the colon of azoxymethane-treated rats (56). Although p21 is a potent regulator of cyclin E- and cyclin D-dependent kinases, we did not detect significant changes in the expression levels of other genes regulating cell growth or survival such as cyclin D, cyclin E, and p27Kip1. The lack of significant changes in the expression of these genes may be due to the method in which RNA was isolated from the whole lung. Laser microdissection of tumors should be considered in future studies (57).

In summary, these data suggest that high intake of EPA and DHA from fish oil reduces prevalence of NNK-induced lung tumors in mice. This effect might be due to fish oil upregulated expressions of 15-LOX and p21 genes. Future studies should focus on the mechanism of fish oil induced protective effects as well as on the intersection between diet and traditional chemotherapy, building on the cooperative effects seen in xenograft studies (58).

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REFERENCES

- MacLean CH, Newberry SJ, Mojica WA, Khanna P, Issa AM, et al.: Effects of omega-3 fatty acids on cancer risk: a systematic review. *J Amer Med Assoc* **295**, 403–415, 2006.
- Simopoulos AP: Omega-3 fatty acids in health and disease and in growth and development. *Am J Clin Nutr* **54**, 438–463, 1991.
- Ruxton CH, Reed SC, Simpson MJ, and Millington KJ: The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *J Hum Nutr Diet* **20**, 275–285, 2007.
- Geelen A, Schouten JM, Kamphuis C, Stam BE, Burema J, et al.: Fish consumption, n-3 fatty acids, and colorectal cancer: a meta-analysis of prospective cohort studies. *Am J Epidemiol* **166**, 1116–1125, 2007.
- Hall MN, Chavarro JE, Lee IM, Willett WC, and Ma J: A 22-year prospective study of fish, n-3 fatty acid intake, and colorectal cancer risk in men. *Cancer Epidemiol Biomarkers Prev* **17**, 1136–1143, 2008.
- Zhang J, Temme EH, and Kesteloot H: Fish consumption is inversely associated with male lung cancer mortality in countries with high levels of cigarette smoking or animal fat consumption. *Int J Epidemiol* **29**, 615–621, 2000.
- Koo LC: Dietary habits and lung cancer risk among Chinese females in Hong Kong who never smoked. *Nutr Cancer* **11**, 155–172, 1988.
- Pierce RJ, Kune GA, Kune S, Watson LF, Field B, et al.: Dietary and alcohol intake, smoking pattern, occupational risk, and family history in lung cancer patients: results of a case-control study in males. *Nutr Cancer* **12**, 237–248, 1989.
- Veierod MB, Laake P, and Thelle DS: Dietary fat intake and risk of lung cancer: a prospective study of 51,452 Norwegian men and women. *Eur J Cancer Prev* **6**, 540–549, 1997.
- Takezaki T, Inoue M, Kataoka H, Ikeda S, Yoshida M, et al.: Diet and lung cancer risk from a 14-year population-based prospective study in Japan: with special reference to fish consumption. *Nutr Cancer* **45**, 160–167, 2003.
- Kvale G, Bjelke E, and Gart JJ: Dietary habits and lung cancer risk. *Int J Cancer* **31**, 397–405, 1983.
- Ozasa K, Watanabe Y, Ito Y, Suzuki K, Tamakoshi A, et al.: Dietary habits and risk of lung cancer death in a large-scale cohort study (JACC Study) in Japan by sex and smoking habit. *Jpn J Cancer Res* **92**, 1259–1269, 2001.
- Okuyama H, Kobayashi T, and Watanabe S: Dietary fatty acids—the N-6/N-3 balance and chronic elderly diseases: excess linoleic acid and relative N-3 deficiency syndrome seen in Japan. *Prog Lipid Res* **35**, 409–457, 1996.
- Xia S-H, Wang J, and Kang JX: Decreased n-6/n-3 fatty acid ratio reduces the invasive potential of human lung cancer cells by downregulation of cell adhesion/invasion-related genes. *Carcinogenesis* **26**, 779–784, 2005.
- Xia S, Lu Y, Wang J, He C, Hong S, et al.: Melanoma growth is reduced in fat-1 transgenic mice: impact of omega-6/omega-3 essential fatty acids. *Proc Natl Acad Sci USA* **103**, 12499–12504, 2006.
- Hoffmann D, Rivenson A, Abbi R, and Wynder EL: A study of tobacco carcinogenesis: effect of the fat content of the diet on the carcinogenic activity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 rats. *Cancer Res* **53**, 2758–2761, 1993.
- Larsson SC, Kumlin M, Ingelman-Sundberg M, and Wolk A: Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am J Clin Nutr* **79**, 935–945, 2004.
- Jump DB: Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr Opin Lipidol* **13**, 155–164, 2002.
- Calder PC and Grimble RF: Polyunsaturated fatty acids, inflammation and immunity. *Eur J Clin Nutr* **56**(3 Suppl), S14–S19, 2002.
- Grimble RF and Tappia PS: Modulation of pro-inflammatory cytokine biology by unsaturated fatty acids. *Zeitschrift für Ernährungswissenschaft* **37**(1 Suppl), 57–65, 1998.
- Grimble RF: Nutritional influences on inflammation. *Nestle Nutrition Workshop Series Clinical & Performance Program* **2**, 63–81, 1999.
- Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, et al.: Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. *J Lipid Res* **44**, 479–486, 2003.

23. Jia Q, Zhou HR, Bennink M, and Pestka JJ: Docosahexaenoic acid attenuates mycotoxin-induced immunoglobulin A nephropathy, interleukin-6 transcription, and mitogen-activated protein kinase phosphorylation in mice. *J Nutr* **134**, 3343–3349, 2004.
24. Yam D, Peled A, Huszar M, and Shinitzky M: Dietary fish oil suppresses tumor growth and metastasis of Lewis lung carcinoma in mice. *Nutr Biochem* **8**, 619–622, 1997.
25. Hecht SS, Morse MA, Amin S, Stoner GD, Jordan KG, et al.: Rapid single-dose model for lung tumor induction in A/J mice by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the effect of diet. *Carcinogenesis* **10**, 1901–1904, 1989.
26. Mernitz H, Smith DE, Zhu AX, and Wang X-D: 9-cis Retinoic acid inhibition of lung carcinogenesis in the A/J mouse model is accompanied by increased expression of RAR-beta but no change in cyclooxygenase-2. *Cancer Lett* **244**, 101–108, 2006.
27. Mernitz H, Smith DE, Wood RJ, Russell RM, and Wang X-D: Inhibition of lung carcinogenesis by 1alpha,25-dihydroxyvitamin D3 and 9-cis retinoic acid in the A/J mouse model: evidence of retinoid mitigation of vitamin D toxicity. *Int J Cancer* **120**, 1402–1409, 2007.
28. Lian F, Smith DE, Ernst H, Russell RM, and Wang XD: Apo-10'-lycopenoic acid inhibits lung cancer cell growth in vitro, and suppresses lung tumorigenesis in the A/J mouse model in vivo. *Carcinogenesis* **28**, 1567–1574, 2007.
29. Castonguay A and Rioux N: Inhibition of lung tumorigenesis by sulindac: comparison of two experimental protocols. *Carcinogenesis* **18**, 491–496, 1997.
30. Shimkin MB and Stoner GD: Lung tumors in mice: application to carcinogenesis bioassay. *Adv Cancer Res* **21**, 1–58, 1975.
31. Yam D, Peled A, and Shinitzky M: Suppression of tumor growth and metastasis by dietary fish oil combined with vitamins E and C and cisplatin. *Cancer Chemother Pharmacol* **47**, 34–40, 2001.
32. de Bravo MG, de Antueno RJ, Toledo J, De Tomas ME, Mercuri OF, et al.: Effects of an eicosapentaenoic and docosahexaenoic acid concentrate on a human lung carcinoma grown in nude mice. *Lipids* **26**, 866–870, 1991.
33. Burdge G: Alpha-linolenic acid metabolism in men and women: nutritional and biological implications. *Curr Opin Clin Nutr Metab Care* **7**, 137–144, 2004.
34. Bougnoux P, Maillard V, and Chajes V: Omega-6/omega-3 polyunsaturated fatty acids ratio and breast cancer. *World Rev Nutr Diet* **94**, 158–165, 2005.
35. Jude S, Roger S, Martel E, Besson P, Richard S, et al.: Dietary long-chain omega-3 fatty acids of marine origin: a comparison of their protective effects on coronary heart disease and breast cancers. *Prog Biophys Mol Biol* **90**, 299–325, 2006.
36. Bartsch H, Nair J, and Owen RW: Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis* **20**, 2209–2218, 1999.
37. Roynette CE, Calder PC, Dupertuis YM, and Pichard C: n-3 polyunsaturated fatty acids and colon cancer prevention. *Clin Nutr* **23**, 139–151, 2004.
38. Willett WC: Specific fatty acids and risks of breast and prostate cancer: dietary intake. *Am J Clin Nutr* **66**, 1557S–1563S, 1997.
39. Astorg P: Dietary N-6 and N-3 polyunsaturated fatty acids and prostate cancer risk: a review of epidemiological and experimental evidence. *Cancer Causes Control* **15**, 367–386, 2004.
40. Furstenberger G, Krieg P, Muller-Decker K, and Habenicht AJ: What are cyclooxygenases and lipoxygenases doing in the driver's seat of carcinogenesis? *Int J Cancer* **119**, 2247–2254, 2006.
41. Shureiqi I, Wu Y, Chen D, Yang XL, Guan B, et al.: The critical role of 15-lipoxygenase-1 in colorectal epithelial cell terminal differentiation and tumorigenesis. *Cancer Res* **65**, 11486–11492, 2005.
42. Nixon JB, Kim KS, Lamb PW, Bottone FG, and Eling TE: 15-Lipoxygenase-1 has anti-tumorigenic effects in colorectal cancer. *Prostaglandins Leukot Essent Fatty Acids* **70**, 7–15, 2004.
43. Hennig R, Kehl T, Noor S, Ding XZ, Rao SM, et al.: 15-lipoxygenase-1 production is lost in pancreatic cancer and overexpression of the gene inhibits tumor cell growth. *Neoplasia (New York)* **9**, 917–926, 2007.
44. Kim JS, Baek SJ, Bottone FG Jr, Sali T, and Eling TE: Overexpression of 15-lipoxygenase-1 induces growth arrest through phosphorylation of p53 in human colorectal cancer cells. *Mol Cancer Res* **3**, 511–517, 2005.
45. Shureiqi I, Chen D, Lotan R, Yang P, Newman RA, et al.: 15-Lipoxygenase-1 mediates nonsteroidal anti-inflammatory drug-induced apoptosis independently of cyclooxygenase-2 in colon cancer cells. *Cancer Res* **60**, 6846–6850, 2000.
46. Wu J, Xia HH, Tu SP, Fan DM, Lin MC, et al.: 15-Lipoxygenase-1 mediates cyclooxygenase-2 inhibitor-induced apoptosis in gastric cancer. *Carcinogenesis* **24**, 243–247, 2003.
47. Ding XZ, Tong WG, and Adrian TE: 12-lipoxygenase metabolite 12(S)-HETE stimulates human pancreatic cancer cell proliferation via protein tyrosine phosphorylation and ERK activation. *Int J Cancer* **94**, 630–636, 2001.
48. Gao X, Grignon DJ, Chbihi T, Zacharek A, Chen YQ, et al.: Elevated 12-lipoxygenase mRNA expression correlates with advanced stage and poor differentiation of human prostate cancer. *Urology* **46**, 227–237, 1995.
49. Natarajan R, Esworthy R, Bai W, Gu JL, Wilczynski S, et al.: Increased 12-lipoxygenase expression in breast cancer tissues and cells: regulation by epidermal growth factor. *J Clin Endocrinol Metab* **82**, 1790–1798, 1997.
50. Wong BC, Wang WP, Cho CH, Fan XM, Lin MC, et al.: 12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells. *Carcinogenesis* **22**, 1349–1354, 2001.
51. Piao YS, Du YC, Oshima H, Jin JC, Nomura M, et al.: Platelet-type 12-lipoxygenase accelerates tumor promotion of mouse epidermal cells through enhancement of cloning efficiency. *Carcinogenesis* **29**, 440–447, 2008.
52. El-Bayoumy K, Iatropoulos M, Amin S, Hoffmann D, and Wynder EL: Increased expression of cyclooxygenase-2 in rat lung tumors induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanone: the impact of a high-fat diet. *Cancer Res* **59**, 1400–1403, 1999.
53. Razani-Boroujerdi S and Sopori ML: Early manifestations of NNK-induced lung cancer: role of lung immunity in tumor susceptibility. *Am J Respir Cell Mol Biol* **36**, 13–19, 2007.
54. Ye B, Zhang YX, Yang F, Chen HL, Xia D, et al.: Induction of lung lesions in Wistar rats by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and its inhibition by aspirin and phenethyl isothiocyanate. *BMC Cancer* **7**, 90, 2007.
55. Shoji T, Tanaka F, Takata T, Yanagihara K, Otake Y, et al. Clinical significance of p21 expression in non-small-cell lung cancer. *J Clin Oncol* **20**, 3865–3871, 2002.
56. Crim KC, Sanders LM, Hong MY, Taddeo SS, Turner ND, et al.: Upregulation of p21Waf1/Cip1 expression in vivo by butyrate administration can be chemoprotective or chemopromotive depending on the lipid component of the diet. *Carcinogenesis* **29**, 1415–1420, 2008.
57. Narayanan BA, Narayanan NK, and Reddy BS: Docosahexaenoic acid regulated genes and transcription factors inducing apoptosis in human colon cancer cells. *Int J Oncol* **19**, 1255–1262, 2001.
58. Hardman WE, Moyer MP, and Cameron IL: Dietary fish oil sensitizes A549 lung xenografts to doxorubicin chemotherapy. *Cancer Lett* **151**, 145–151, 2000.